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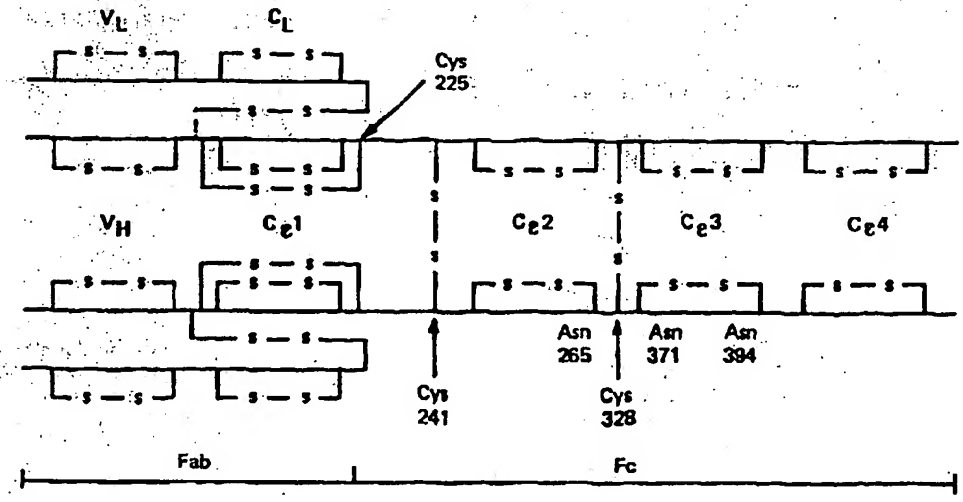


(57) Abstract

The invention provides a mutated glycosylated polypeptide which includes at least a part of the hIgE-Fc chain of sufficient length to bind to Fc$\varepsilon$ RI and/or Fc$\varepsilon$ RII receptor sites on human cells wherein Cys 225 has been mutated by replacement with another amino acid residue or has been deleted, optionally together with Val 224 and Ser 226 or with Val 224 Ser 226 and Arg 227, and wherein at least one of the sites Asn 394 and, if present, Asn 265 and/or Asn 371 bears a glycoside chain.

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# MUTATED HIG E FRAGMENTS AND DERIVATIVE THEREOF

This invention relates to polypeptides, more particularly to polypeptide competitors for IgE receptor sites on cells.

Antibodies of the immunoglobulin E (IgE) class make up a minute proportion (ca 0.01%) of the total immunoglobulin in normal human serum. However, their activities are powerfully amplified by the cell receptors to which they bind, and elevated levels of IgE play a central role in atopic allergy. The biological activities of immunoglobulin E (IgE) depend on its interaction with two receptors, FcεRI and FcεRII, expressed on effector cells; cross-linking of surface receptor-bound IgE allows antigen triggering of cell activation and is implicated in the aetiology of allergic diseases. Antigens bind to the Fab regions of the antibody, while the receptors bind to the Fc region, comprising a dimer of the three C-terminal domains of the ε chain, comprising the second, third and fourth constant region domains (Cε2-Cε4).

Cross-linking of IgE bound to the "high affinity" receptor, FcεRI, on mast cells and basophils leads to cell degranulation and the release of pre-formed and newly synthesised mediators, responsible for immediate hypersensitivity and for initiating a leucocyte cascade that results in a later inflammatory response. This has been reported by *Ishizaka et al., Immunochemistry 7:687 (1970)* and *Prog. Allergy 19:60 (1975)*. The so-called "low affinity" receptor, FcεRII, has many activities, including the sensitisation of inflammatory cells for IgE antibody-dependent reactions, as reported by *Capron et al., Immunol. Today, 7:15 (1986)*, and IgE-dependent antigen presentation to T cells by B cells. This latter activity is reviewed in a paper by *Delespesse et al, Adv. Immunol. 49:149 (1991)*.

It has been reported by *Ishizaka et al,*

*Immunochemistry 7:687 (1970)* and by *Stanworth et al., Lancet 2:17 (1968)* that IgE is cleaved by papain into Fab and Fc fragments, homologous to those that result from the fragmentation of IgG. These authors also report that cell receptors recognise the Fc fragment, a disulphide-bridged dimer of the three carboxy-terminal domains (Cε2-Cε4) of each ε-chain, covalently linked by two disulphide bonds between the pairs of Cys 328 and Cys 241 of the Cε2 residues. Evidence to this effect is also provided by *Spiegelberg, Adv. in Immunol. 35:61 (1984)*, as well as by *Dorrington et al., Immunol. Rev. 41:3 (1978)* and *Helm et al., Eur. J. Immunol. 21:1543 (1991)*. IgE is heavily N-glycosylated, containing 13% by weight of carbohydrate, as compared with 3% for IgG according to *Dorrington et al., Immunol. Rev. 41:3 (1978)*. The IgE-Fc is potentially N-glycosylated at Asn 265 (Cε2 domain), Asn 371, Asn 383, and Asn 394 (all three in the Cε3 domain). The last of these is homologous to the glycosylation site in the corresponding domains of IgG, IgM and IgD (C 2, Cμ3, Cδ2). Its predicted position is on the apposed faces of the Cε3 domains, which are expected to resemble the C 2 domains of IgG in their relative orientation according to *Deisenhofer, Biochemistry 20:2361 (1981)* and *Sutton et al., Biochem. Soc. Trans. 11:130 (1983)*. By contrast, carbohydrate chains linked to Asn 265, Asn 383 and Asn 371 are predicted to be fully exposed on the outer surface of the molecule, and are specific for IgE.

Recombinant human IgE Fc (hIgE-Fc) has been expressed in E. coli, as taught by *Kenten et al., Proc. Natl Acad. Sci. USA 81:2955 (1984)* and *Liu et al., Proc. Natl. Acad. Sci. USA 81:5369 (1984)*. This product binds to FcεRI with the same affinity as myeloma IgE(PS) according to *Ishizaka et al., Proc. Natl. Acad. Sci. USA 83:8323 (1986)*; since the E. coli product is non-glycosylated, these results

imply that carbohydrate does not form a part of the binding site for FcεRI. In contrast to FcεRI and all other immunoglobulin receptors which belong to the immunoglobulin superfamily, FcεRII is a member of the C-type lectin family, and it was therefore expected that it might recognise the carbohydrate moiety of IgE. This is clearly not the case, however, since the E. coli hIgE-Fc binds to the receptor with about the same affinity as native IgE; in this connection reference may be made to *Vercelli, Nature 338:649 (1989)*. In IgG, glycosylation of Asn 297 (the homologue of Asn 394 in IgE) is required for IgG to bind with native affinity to its IgG-Fc receptors according to *Nose et al, Proc. Natl. Acad. Sci. USA 80:6632 (1983)*, to *Leatherbarrow et al., Mol. Immunol. 22:407 (1985)* and to *Heyman et al., J. Immunol. 134:4018 (1985)*, but this could be due to an indirect effect upon the polypeptide conformation.

The purification and characterisation of human recombinant IgE-Fc fragments that bind to the human high affinity IgE receptor is described by *Basu et al., J. Biol. Chem. 269:13118 (1993)*. These authors state that the smallest IgE fragment that showed FcεRIα binding activity spans amino acids 329-547 and lacks the entire Cε2 domain. They also reported two active fragments, viz. Fcε(315-547) and Fcε(329-547), which were overexpressed in Chinese hamster ovary (CHO) cells and purified to homogeneity. The presence of N-linked glycosylation was detected in both proteins.

It is possible to obtain high levels of expression of the recombinant human IgE-Fc (hIgE-Fc) in E. coli, but not to recover more than a small fraction of the desired product, a dimer of ε-chain fragments with the same affinity for receptors as native IgE; this can be attributed to incorrect folding and/or aggregation of the bulk of the material in vitro.

The structure of human immunoglobulin E (hIgE) was first determined by *Bennich et al., Progress in Immunology II, Vol I:49 (1974)*. In later work, described in *Bennich et al., Int. Arch. Allergy Appl. Immunol. 53:459 (1977)*, it was reported that amino acid 322 is asparagine and not aspartic acid. A corrected formula appears in EP-A-0102634.

The hIgE-Fc contains four potential glycosylation sites, at Asn 265 (in Cε2) and Asn 371, Asn 383 and Asn 394 (in Cε3). Three of these, Asn 265, Asn 371 and Asn 383, are predicted to be on the external surface of the protein according to *Helm et al, Eur. J. Immunol., 21:1543* (1991), while Asn 394, being homologous to Asn 297 in IgG is predicted to be partially buried in the protein. It has also been reported that the glycosylation site at Asn 383 is not occupied in the myeloma IgE from a patient (designated "patient ND"), whose expressed ε-chain cDNA sequence is immortalised in the hIgE-Fc expression constructs reported by *Kenten et al, Proc. Natl. Acad. Sci. USA 81:2955* (1984).

In WO-A-88/00204 there is described a polypeptide residue with a chain length of 76 amino acid residues which is a competitor for hIgE and binds specifically to the so called "high affinity" Fc receptor sites for IgE (i.e. FcεRI sites) which exist on human cells, particularly mast cells and basophils.

A larger polypeptide chain which binds to so called "low affinity" receptor (or FcεRII) sites is disclosed in WO-A-89/04834.

US-A-4171299 and US-A-4161522 disclose that an oligopeptide containing from three to ten amino acids in a sequence selected from a portion of amino acids 265 to 537, according to the Bennich nomenclature, of the Fc region of hIgE will block Fc receptors of mast cells.

It is an object of the present invention to provide novel synthetic polypeptides, related to portions of the

hIgE molecule, in particular to at least a part of the hIgE-Fc fragment, which are useful in the study and treatment of allergy conditions. It is a further object of the present invention to provide synthetic mutant polypeptides derived from the Fc chain of hIgE which do not form aggregates larger than dimers. Yet a further object of the present invention is to provide synthetic polypeptides which bear glycoside side chains on at least one of the available sites of the Fc chain of hIgE and which are useful for investigation of and amelioration of allergic conditions. The invention also has for an object the provision of mutant polypeptides related to the Fc chain of hIgE which have one or more of the glycosylation sites present on the Fc chain of hIgE substituted by an amino acid residue which cannot be glycosylated.

According to the present invention there is provided a mutated glycosylated polypeptide which includes at least a part of the hIgE-Fc chain of sufficient length to bind to Fc$\epsilon$RI and/or Fc$\epsilon$RII receptor sites on human cells wherein Cys 225 has been mutated by replacement with another amino acid residue or has been deleted, optionally together with Val 224 and Ser 226 or with Val 224 Ser 226 and Arg 227, or with Val 224 Ser 226 Arg 227 and Asp 228, and wherein at least one of the sites Asn 394 and, if present, Asn 265 and/or Asn 371 bears a glycoside chain.

In this specification the numbering of the amino acid residues, except where otherwise indicated, is based upon that of *Dorrington et al., Immunological Reviews, 41:3 (1978)* at page 7, as modified by the results of subsequent research. Specifically the numbering of the amino acid residues is as follows:-

[SEQ ID No: 1]

```
224                                                          VC
226  SRDFTPPTVK ILQSSCDGGG HFPPTIQLLC LVSGYTPGTI NITWLEDGQV
275  MDVDLSTAST TQEGELASTQ SELTLSQKHW LSDRTYTCQV TYQGHTFEDS
325  TKKCADSNPR GVSAYLSRPS PFDLFIRKSP TITCLVVDLA PSKGTVNLTW
375  SRASGKPVNH STRKEEKQRN GTLTVTSTLP VGTRDWIEGE TYQCRVTHPH
425  LPRALMRSTT KTSGPRAAPE VYAFATPEWP GSRDKRTLAC LIQNFMPEDI
475  SVQWLHNEVQ LPDARHSTTQ PRKTKGSGFF VFSRLEVTRA EWEQKDEFIC
525  RAVHEAASPS QTVQRAVSVN PGK
```

In this sequence the amino acid residues are identified by the usual one-letter symbols:-

| Amino acid | Three-letter abbreviation | One-letter symbol |
|---|---|---|
| Alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid | Asp | D |
| Asparagine or aspartic acid | Asx | B |
| Cysteine | Cys | C |
| Glutamine | Gln | Q |
| Glutamic acid | Glu | E |
| Glutamine or glutamic acid | Glx | Z |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |

The leucine residue (L) identified by an asterisk (*) is assigned the number 253A in the above sequence. The underlined residues are Cys 225, Asn 265, Asn 371 and Asn 394.

The DNA sequence coding for this sequence is set out below:-

[SEQ ID No: 2]

GTCTGCTC CAGGGACTTC ACCCCGCCCA CCGTGAAGAT
CTTACAGTCG TCCTGCGACG GCGGCGGGCA CTTCCCCCCG ACCATCCAGC
TCCTGTGCCT CGTCTCTGGG TACACCCCAG GGACTATCAA CATCACCTGG
CTGGAGGACG GGCAGGTCAT GGACGTGGAC TTGTCCACCG CCTCTACCAC
GCAGGAGGGT GAGCTGGCCT CCACACAAAG CGAGCTCACC CTCAGCCAGA
AGCACTGGCT GTCAGACCGC ACCTACACCT GCCAGGTCAC CTATCAAGGT
CACACCTTTG AGGACAGCAC CAAGAAGTGT GCAGGTACGT TCCCACCTGC
CCTGGTGGCC GCCACGGAGG CCAGAGAAGA GGGGCGGGTG GGCCTCACAC
AGCCCTCCGG TGTACCACAG ATTCCAACCC GAGAGGGGTG AGCGCCTACC
TAAGCCGGCC CAGCCCGTTC GACCTGTTCA TCCGCAAGTC GCCCACGATC
ACCTGTCTGG TGGTGGACCT GGCACCCAGC AAGGGGACCG TGAACCTGAC
CTGGTCCCGG GCCAGTGGGA AGCCTGTGAA CCACTCCACC AGAAAGGAGG
AGAAGCAGCG CAATGGCACG TTAACCGTCA CGTCCACCCT GCCGGTGGGC
ACCCGAGACT GGATCGAGGG GGAGACCTAC CAGTGCAGGG TGACCCACCC
CCACCTGCCC AGGGCCCTCA TGCGGTCCAC GACCAAGACC AGCGGTGAGC
CATGGGCAGG CCGGGGTCGT GGGGGAAGGG AGGGAGCGAG TGAGCGGGGC
CCGGGCTGAC CCCACGTCTG GCCACAGGCC CGCGTGCTGC CCCGGAAGTC
TATGCGTTTG CGACGCCGGA GTGGCCGGGG AGCCGGGACA AGCGCACCCT
CGCCTGCCTG ATCCAGAACT TCATGCCTGA GGACATCTCG GTGCAGTGGC
TGCACAACGA GGTGCAGCTC CCGGACGCCC GGCACAGCAC GACGCAGCCC
CGCAAGACCA AGGGCTCCGG CTTCTTCGTC TTCAGCCGCC TGGAGGTGAC
CAGGGCCGAA TGGGAGCAGA AAGATGAGTT CATCTGCCGT GCAGTCCATG
AGGCAGCGAG CCCCTCACAG ACCGTCCAGC GAGCGGTGTC TGTAAATCCC
GGTAAATGAC GTACTCCTGC CTCCCTCCCT CCCAGGGCTC CATCCAGCTG
TGCAGTGGGG AGGACTGGCC AGACCTTCTG TCCACTGTTG CAATGACCCC
AGGAAGCTAC CCCCAATAAA CTGTGCCTGC TCAGAGCCCC AGTACACCCA
TTCTTGGGAG CGGGCAGGGC.

The codons in this DNA sequence where mutations are introduced in order to produce the polypeptides according to the invention are underlined in the sequence. For the mutation wherein Cys 225 is replaced by Ala the corresponding codon TGC is altered to GCC. In the case of the mutations of Asn 265 and Asn 371 to Gln the

corresponding codon AAC is in each case changed to CAG.

The invention further provides a polypeptide which binds to human immunoglobulin E (hIgE) receptor sites on cells and which is of the formula:

$AA_n$-hIgE-Fc (W225, X265, Y371, Z394)

wherein:

AA represents an amino acid residue which may be the same as or different from any other group AA which may be present in the molecule;

n represents zero or an integer from 1 to about 10; and

hIgE-Fc (W225, X265, Y371, Z394) represents a mutant version of the hIgE-Fc chain with a mutation or deletion at least at position 225; wherein

W225 represents deletion of Val 224 and Cys 225, of Val 224, Cys 225 and Ser 226, or of Val 224, Cys 225, Ser 226 and Arg 227, or of Val 224, Cys 225, Ser 226, Arg 227 and Asp 228, or represents the residue of an amino acid at position 225 other than cysteine;

X265 is the residue of an amino acid at position 265;

Y371 is the residue of an amino acid at position 371; and

Z394 is the residue of an amino acid at position 394; and wherein at least one of X265, Y371 and Z394 may be an asparagine residue which may be glycosylated;

or a fragment of such a polypeptide which lacks up to 10 terminal amino acid residues of the Cε4 domain at the carboxy end of the chain.

In such a polypeptide W225 may represent an alanine residue. In a particularly preferred polypeptide X265 represents a glutamine residue. Y371 may represent a glutamine residue, while Z394 may represent a glutamine residue.

Preferred polypeptides are selected from:

$AA_n$-hIgE-Fc (Ala 225)

$AA_n$-hIgE-Fc (Ala 225, Gln 265)

$AA_n$-hIgE-Fc (Ala 225, Gln 371)

$AA_n$-hIgE-Fc (Ala 225, Gln 394)

$AA_n$-hIgE-Fc (Ala 225, Gln 265, Gln 371)

$AA_n$-hIgE-Fc (Ala 225, Gln 265, Gln 394)

$AA_n$-hIgE-Fc (Ala 225, Gln 371, Gln 394)

$AA_n$-hIgE-Fc (Ala 225, Gln 265, Gln 371, Gln 394).

In the above formula $AA_n$ preferably represents an inert polypeptide sequence, for example Asp-Ile.

In the polypeptide the N-terminal sequence of the group hIgE-Fc (W225, X265, Y371, Z394) may have the structure:

[SEQ ID No: 3]

Asp Ile Val Ala Ser Xaa Asp Phe Thr

where Xaa is the residue of an amino acid, for example an arginine residue. In this case $AA_n$ represents Asp-Ile and the Ala residue in this sequence is the residue replacing Cys 225.

W225 may alternatively represent deletion of Val 224 and Cys 225, or of Val 224 Cys 225 Ser 226, or of Val 224 Cys 225 Ser 226 Arg 227, or of Val 224 Cys 225 Ser 226 Arg 227 Asp 228.

The invention also provides a vector containing cDNA coding for a polypeptide according to the invention, as well as a mammalian cell line, e.g. a human cell line or a Chinese hamster ovary cell line, containing DNA coding for a polypeptide according to the invention, which expresses such a polypeptide.

For convenience the mutant version of hIgE-Fc (Ala 225) with the additional sequence Asp-Ile at the amino end of the chain is referred to as X'-hIgE, while the corresponding double and triple mutants are also given the

prefix X'-hIgE.

Included vectors according to the invention are expression vectors that lead to secretion of X'-hIgE-Fc, and the mutants X'-hIgE-Fc (Gln 265), X'-hIgE-Fc (Gln 371), and X'-hIgE-Fc (Gln 265, Gln 371), from mammalian cells at levels up to 100 mg/litre of culture.

Also provided according to the present invention are pharmaceutical preparations comprising a polypeptide according to the invention and a carrier therefor. Such carriers are well known in the art.

The invention provides a novel method for the production of a dimeric immunoglobulin Fc chain fragment by expression in, and secretion from, mammalian cells. Secretion is brought about by linking the DNA sequence encoding the Fc fragment of the human X'-IgE-Fc(hIgE-Fc) or a mutant thereof to a kappa-light chain signal sequence. The recombinant DNA can be cloned into mammalian expression vectors for transfection of CHO (Chinese hamster ovary) or NS-O cells. The X'-hIgE-Fc chain or mutant thereof is assembled into dimers, correctly processed, and secreted from the CHO cells. Similar results are obtained when the gene is transfected into the myeloma cell line NS-O, showing that the X'-hIgE-Fc and its mutants may be produced in a variety of mammalian cells.

The secreted wild type X'-hIgE-Fc contains the full sequence of Cε2-Cε4 with one amino acid substitution, alanine for cysteine at position 225, and an extension of two amino acids (aspartic acid and isoleucine), which remain at the N-terminus after cleavage of the leader signal sequence from the precursor peptide. The replacement of Cys 225 by alanine is believed to be an important feature in the design of the X'-hIgE-Fc and its mutants for secretion by mammalian cells. The two free cysteine residues are predicted to lie apart from each other, but available for

reaction with other molecules, on the surface of the Fc fragment and hence available to mediate the formation of aggregates.

Recombinant X'-hIgE-Fc can also be prepared by expression in E. coli. The product accumulates in the bacterium as an insoluble inclusion body, requiring dispersal in denaturing solvents. The recovery of native structure therefore involves correct folding, assembly of dimers and disulphide bond formation. The recovery of recombinant X'-hIgE-Fc after the application of these procedures is at best about 5-10%, although the biological activity of the final product is indistinguishable from native IgE. This low recovery may reflect the proportion of incorrectly folded and/or disulphide-bonded fragments which are eliminated in the purification of the active material. No evidence for the secretion of inactive products was seen in the production of the polypeptides according to the invention when secreted from CHO or NS-0 cells. The total amount of X'-hIgE-Fc synthesised in CHO cells is less than in E. coli (2mg/litre in CHO cells, as against > 55 mg/litre in E. coli), but with NS-0 cells expression is increased to a level (ca 100 mg/litre) higher than that obtained in E. coli.

A secretion system has been reported by *Kitai et al., Appl. Microbiol. Biotechnol. 28:52*, for the expression of assembled IgG-Fc in E. coli, but the yield was lower (3 mg/litre of culture) than from non-secreting bacterial expression systems. This is comparable to the level of X'-hIgE-Fc secretion from CHO cells achievable in accordance with the invention (i.e. approximately 2 mg/litre of culture supernatant), but greatly inferior to the amount accumulated by secretion from the NS-0 cell line.

In contrast to bacterial expression, the mammalian expression system allows production of glycosylation site

mutants. X'-hIgE-Fc has been prepared with different combinations of the surface carbohydrate chains of the IgE-Fc fragment. Analysis of transient expression products reveals that Asn 265 was completely glycosylated, but that Asn 371 is rarely glycosylated, and Asn 383 not at all, in CHO cells. The establishment of permanent lines expressing the mutant type X'-hIgE-Fc and the triple mutant, X'-hIgE-Fc(Gln 265, Gln 371), provides material for biological assays.

The sensitisation of human basophils by X'-hIgE-Fc and X'-hIgE-Fc(Gln 265, Gln 371) provides an assay for effector function. Basophils sensitised by each X'-IgE-Fc fragment or IgE show almost identical response to anti-IgE stimulation. The small shift in the curve of histamine release from IgE(PS)-sensitised cells is presumably due to differences between the interactions of IgE an IgE-Fc fragments with the polyclonal anti-IgE, which was raised against whole myeloma IgE. These results indicate that both wild type and mutant recombinant hIgE-Fc are as effective as whole IgE in sensitising FcεRI of human basophils for histamine release.

Comparison of wild type X'-hIgE-Fc and mutant products reveals that Asn 371 is rarely glycosylated in Chinese hamster ovary cells. Both the double mutant, X'-hIgE-Fc (Gln 265, Gln 371), and wild type, X'-hIgE-Fc, bind to the high affinity IgE receptor, FcεRI, with about the same affinity as myeloma IgE ($K_a$ in the range $10^{10}$-$10^{11}$ $M^{-1}$) and were able to sensitise isolated human basophils for anti-IgE triggering of histamine release. However, only the double mutant, X'-hIgE-Fc (Gln 265, Gln 371), approached the affinity of myeloma LgE for the low affinity receptor, FcεRII ($K_a$ = 7.3 x $10^7$ $M^{-1}$), whereas the wild type, X'-hIgE-Fc, bound with a ten-fold lower affinity to the low affinity receptor ($K_a$ = 4.1 x $10^6$ $M^{-1}$).

In the drawings:-

Figure 1 is a diagrammatic representation of the hIgE molecule, indicating the Fab and Fc fragments and the Cε1 to Cε4 domains, as well as the intermolecular and intramolecular disulphide linkages;

Figure 2 is a representation of the structural relationship between part of the natural hIgE-Fc chain and its associated DNA sequence;

Figure 3 is a similar representation of the corresponding part of a mutation with Cys 225 replaced by Ala 225, i.e. X'-hIgE-Fc (Ala 225), and its associated mutated DNA sequence;

Figure 4 is a similar representation of part of a mutation with Asn 265 replaced by Gln 265, i.e. X'-hIgE-Fc (Ala 225, Gln 265), and its associated mutated DNA sequence;

Figure 5 is a similar representation of part of a mutation with Asn 371 replaced by Gln 371, i.e. X'-hIgE-Fc (Ala 225, Gln 371), and its associated DNA sequence;

Figure 6 is a restriction map illustrating construction of a mammalian expression vector for the polypeptides of the invention;

Figure 7 shows SDS-polyacrylamide gel electrophoresis patterns of various polypeptides which are mutations of the hIgE-Fc chain both under reducing and non-reducing conditions;

Figure 8 shows similar patterns for purified wild type hIgE-Fc and the triple mutation site mutant X'-hIgE-Fc (Ala 225, Gln 265, Gln 371); and

Figure 9 illustrates the effector function of hIgE-Fc.

In more detail Figure 1 shows the covalent structure of human IgE. The locations of intra-chain and inter-chain disulphide bonds (S-S) in the variable (V) and four constant (Cε1, Cε2, Cε3 and Cε4) domains are shown

schematically together with the extent of the Fc chain. The arrangement of the two inter-chain bonds at Cys 241 and Cys 328 is shown parallel. The position of Cys 225, which has been mutated to alanine in the X'-hIgE-Fc, and of the three glycosylation sites at Asn 265 and Asn 371 and Asn 394, are also indicated.

Figure 2 sets out amino acid and nucleotide listings as follows:

[SEQ ID No: 4]

...KTFSVCSRD...

and

[SEQ ID No: 5]

...AAAACCTTCA GCGTCTGCTC CAGGGAC...

Figure 3 includes amino acid sequences as follows:

[SEQ ID No: 6]

...CDIVASRD...

and

[SEQ ID No: 7]

...TGTGATATCG TCGCCTCCAG GGAC...

respectively.

Although some authors consider that the junction between the Cε1 and Cε2 domains occurs between Cys 225 and Ser 226, for the purposes of the present invention this boundary is taken as occurring where determined by the DNA intron and exon boundaries, in other words between Ser 223 and Val 224. This is illustrated in Figure 2.

The construction and expression of a high expression vector for X'-hIgE-Fc are illustrated in Figure 3 which shows the nucleotide and amino acid sequence at the natural junction between Cε1 and Cε2 of the human ε-chain. The N-terminal portion of the Cε2 domain was reconstructed with synthetic oligonucleotides to include the replacement of Cys 225 by alanine and to allow the incorporation of an EcoRV restriction site at the 5'-end of the X'-hIgE-Fc gene.

A mouse variable kappa-chain leader sequence was ligated to this site. The N-terminal dipeptide sequence (Asp.Ile) of the mature X'-hIgE-Fc derives from the proteolytic cleavage or "processing" of the leader sequence at the position marked with an asterisk (*).

Figures 4 and 5 indicate sequences of mutagenic oligonucleotide pairs. Those of Figure 4, reading from the 5' end are:

[SEQ ID No: 8]

GGGACTATCC AGATCACCTG G

and

[SEQ ID No: 9]

CCAGGTGATC TGGATAGTCC C

reapectively, while those of Figure 5 are:

[SEQ ID No: 10]

GGGACCGTGC AGCTGACCTG G

and

[SEQ ID No: 11]

CCAGGTCAGC TGCACGGTCC C

respectively.

Figure 6 is a map of the expression vector pEE6HCMVgpt (see *Stephens et al., Nucl. Acids Res. 17:7110 (1989)*) into which the X'-hIgE-Fc construct was cloned.

Figure 7 shows SDS-polyacrylamide gel electrophoresis patterns of transiently expressed X'-hIgE-Fc proteins. X'-hIgE-Fc proteins were transiently expressed in biosynthetically labelled L761H cells. Secreted X'-hIgE-Fc proteins were immunoprecipitated from the cell medium with anti-hIgE-Fc mAb 7.12 described by *Sherr et al., J. Immunol. 142:181 (1989)* coupled to Sepharose 4B and was analysed under reducing conditions (lanes A1 to A4) and non-reducing conditions (lanes B1 to B4) on SDS 10% polyacrylamide gels. Lanes A1 and B2 show X'-hIgE-Fc(Gln 265, Gln 371), while lanes A2 and B1 show X'-hIgE-Fc (Gln 371). Lanes A3 and B3

are X'-hIgE-Fc (Gln 265) and lanes A4 and B4 are X'-hIgE-Fc (wild type). The positions of molecular weight (kDa) markers are indicated.

In Figure 8 there are shown SDS-polyacrylamide gel electrophoresis patterns of purified wild type X'-hIgE-Fc and the mutant X'-hIgE-Fc (Gln 265, Gln 371). The X'-hIgE-Fc proteins secreted from mammalian cells were affinity purified on anti-hIgE-Fc mAB 7.12 coupled to Sepharose 4B. Purified samples were electrophoresed under reducing conditions (lanes 1 to 4) and non-reducing conditions (lanes 6 to 8) on a denaturing 10% polyacrylamide gel. A sample of X'-hIgE-Fc expressed in E. coli was also electrophoresed for comparison. Lanes 1 and 6 are X'-hIgE-Fc (wild type); lanes 2 and 7 are X'-hIgE-Fc(Gln 265, Gln 371); lanes 3 and 8 are X'-hIgE-Fc expressed in E. coli; and lane 4 is a mixture of high molecular weight standards (the molecular weights of the individual standards are indicted in kDa).

The binding of X'-hIgE-Fc and mutants thereof was investigated. The fraction of functional X'-hIgE-Fc or mutant thereof and IgE(SF25) was determined from the percentage of molecules bound to an excess of a stable line of CHO cells expressing the human FcεRI(CHO-hFcεRI) described by *Wang et al., J. Exp. Med. 175:1353 (1989)*, using the method of *Isersky et al., J. Immunol. 112:1909 (1974)*. IgE(SF25) is a recombinant chimeric IgE, with a mouse antibody heavy chain variable region and a human epsilon constant region sequence, and a corresponding mouse light chain. The concentrations of the X'-hIgE-Fc, of its mutants, and of IgE(SF25) used in all the assays was corrected for the fraction of functional molecules, typically in the range of 50-85%.

Figure 9 illustrates the effector function of X'-hIgE-Fc proteins. Human basophil leukocytes were passively sensitised with IgE(PS), X'-hIgE-Fc fragments or

buffer and challenged with various concentrations of anti-IgE antibody. Spontaneous release of histamine from basophils sensitised with IgE(PS), wild type X'-hIgE-Fc, X'-hIgE-Fc (Gln 265, Gln 371) or buffer control was 1.7%, 1.9%, 2.1%, 1.7% respectively. The graph shows the percentage of histamine released by sensitised human basophil leukocytes as the concentration of the challenging anti-human IgE antibody was increased. X'-hIgE-Fc (●); X'-hIgE-Fc (Gln 265, Gln 371) (○), IgE(PS) (Δ); non-sensitised (▲).

Mixed leukocytes containing about 1% basophil leukocytes were obtained from 120ml of peripheral blood of a non-atopic healthy donor as described by *Grattan et al., Clin. Exp. Allergy 21:695 (1991)*. The serum IgE level of this donor was less than 1 IU/ml and histamine release from the basophils in response to anti-IgE was 6.1 ± 3.1% (mean ± SD, n = 2), indicating that they were only marginally sensitised with endogenous IgE. The leukocytes were washed twice with HAG (HBS (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4$, pH 7.4) containing 0.03% human serum albumin and 5 mM glucose). The leukocytes were resuspended in 2ml of HAG containing 4 mM EDTA in the presence of human myeloma IgE(PS), X'-hIgE-Fc, X'-hIgE-Fc(Gln 265, Gln 371) or buffer only, followed by incubation at 37°C for 90 minutes with gentle shaking. The cells were washed three times with HAG, resuspended in HAG containing 2mM $CaCl_2$ and 1 mM $MgCl_2$ and challenged with various dilutions of anti-human IgE antibody (goat, ε-chain specific, Sigma, UK), as described by *Grattan et al., Clin. Exp. Allergy 21:695 (1991)*. Briefly, aliquots of sensitised cells (~4 x $10^4$ basophils) were incubated with anti-IgE in a total volume of 200μl for 40 minutes at 37°C. The reaction was stopped by cooling on ice, followed by the addition of 800μl of ice-cold HBS to each tube. The cells were separated from the supernatants following

centrifugation and the histamine content of cell pellets and supernatants was determined by automated fluorometric analysis according to the method of *Siraganian, J. Immunol. Methods 7:283 (1975)*.

The invention is further illustrated in the following Examples.

Example 1

a) Construction of X'-hIgE-Fc Expression Vectors wherein X' is Asp-Ile:

To express X'-hIgE-Fc fragments in mammalian cells, the cDNA sequence encoding the ND myeloma ε-chain Fc of IgE was cloned in a mammalian expression vector. The entire heavy chain gene including the hIgE-Fc sequence was excised from the vector pJJ71 on a HindIII restriction fragment. This vector pJJ71 contains the human ε-chain cDNA cloned from the 266b1 cell line, as reported by *Kenten et al., Proc. Natl. Acad. Sci. USA 79:6661 (1982)*. The fragment was then subcloned, in the opposite orientation, back into the HindIII cut pJJ71 vector. All the sequence coding for the Fc fragment, including all of the Cε2, Cε3 and Cε4 domains, except for 34 bases at the 5'-end, were obtained from a BgIII/BamHI digestion fragment of this vector.

The X'-hIgE-Fc cDNA sequence was adapted for secretion by ligating an EcoRI/EcoRV restriction fragment containing the B72.3 mouse hybridoma kappa-light chain gene leader sequence, as described by *Whittle et al., Protein Eng. 1:499 (1987)*, at the 5'-end of the Fc coding sequence. The light chain gene had previously been mutated to introduce an EcoRV site at the 3'-end of the leader sequence. Such a mutation is silent and allows the leader sequence to be attached to a sequence with an EcoRV 5'-end, while preserving the leader processing site. Comparison of the sequences of the natural Cε1/Cε2 domain and the leader/adapter junctions illustrated in Figures 2 to 5

showed that this construct preserves the proteolytic cleavage, or "processing", site of the leader with minimal alteration of the N-terminus of the hIgE-Fc.

The cysteine at position 225 was also changed to alanine by rebuilding the N-terminal end of the Cε2 DNA sequence with oligonucleotides which include a codon coding for Ala in place of a codon coding for Cys 225. Cys 225 forms an intra-chain disulphide bond with a cysteine in Cε1 in IgE, as shown in Figure 1. Since Cε1 is not part of the Fc, Cys 225 may cause disulphide-linked inter-chain aggregates to form.

For the expression of the X'-hIgE-Fc proteins the entire EcoRI/BamHI leader-hIgE-Fc construct was cloned into EcoRI/BcII-cut pEE6HCMVgpt expression vector, as illustrated in Figure 6, downstream of the strong viral human cytomegalovirus (hCMV) promoter. pEE6HCMVgpt is the major immediate-early promoter-enhancer of hCMV described by *Stephens et al., Nucl. Acids. Res. 17:7110 (1989)*. The resulting vector was propagated in the dam E. coli strain GM242 to prevent methylation of its BcII restriction site.

This construct was further modified by the polymerase chain reaction (PCR) overlap extension method described by *Ho et al., Gene 77:51 (1989)* to introduce glutamine residues in place of the asparagines at positions 265 and 371. Three mutants were made, two with a single substitution at Asn 265 or Asn 371, and a third with substitutions at both positions. The oligonucleotides used in the PCR cloning of these mutants are shown in Figures 4 and 5. The four X'-hIgE-Fc fragments are designated X'-hIgE-Fc (Ala 225) (single mutant or wild type), X'-hIgE-Fc(Ala 225, Gln 265) (double mutant) and X'-hIgE-Fc(Ala 225, Gln 371) (double mutant), and X'-hIgE-Fc(Ala 225, Gln 265, Gln 371) (triple mutant). Confirmation of the mutations, adapter sequence and its functions was carried out by DNA

sequencing using the chain termination procedure of *Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463 (1977)*.

b) Properties of the products of transient expression in CHO (Chinese hamster ovary) cells

Chinese hamster ovary (CHO) cells of the sub-line CHO-L761H described by *Cockett et al., Nucl. Acids Res. 19:319 (1990)* were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1mM L-glutamine, 1 x non-essential amino acids (Gibco, UK) and 10% fetal calf serum (FCS). Each of the four X'-hIgE-Fc protein genes was transfected into the CHO-L761H cells using calcium phosphate co-precipitation by the procedure of *Gorman, DNA Cloning Vol II, Glover, ed. Academic Press, NY, page 413 (1985)*. Newly synthesised proteins were labelled by incubating the transfected cells with [$^{35}$S] methionine (Amersham Int., UK, 100$\mu C_i$/ml at 37°C). Secreted hIgE-Fc was precipitated from the culture supernatants 48h afterwards with the anti-hIgE-Fc mAb 7.12 of *Sherr, J. Immunol. 142:481 (1989)* coupled to Sepharose 4B gel beads (according to the instructions provided by the manufacturer, Pharmacia, UK). The immunoprecipitated material was analysed by electrophoresis on reducing and non-reducing SDS-polyacrylamide gels.

Stable CHO-L761H cell lines were established that secreted either wild type or the 265/371 mutant X'-hIgE-Fc, i.e. X'-hIgE-Fc (Gln 265, Gln 371). 1 x $10^6$ cells growing exponentially in a 9 cm Petri-dish were co-transfected with 10$\mu$g of expression vector containing either the parent type X'-hIgE-Fc(Ala 225), pRY20, or the triple mutant X'-hIgE-Fc(Ala 225, Gln 265, Gln 371), pRY22, and 2$\mu$g of a plasmid, pEE7SalNeo, containing a G418 antibiotic resistance marker, using calcium phosphate co-precipitation. After 24 hours cells were detached from the dish by treatment with trypsin and diluted into 10 ml of growth medium and plated out over

a 96 well microtitre plate using 100µl/well. To each well a further 100µl of growth medium containing 2mg/ml G418 was added. G418-resistant colonies were screened with a hIgE ELISA described below. Positive colonies were picked, expanded and the highest X'-hIgE-Fc secreting cell lines were cloned by limiting dilution.

To scale up the production of X'-hIgE-Fc proteins, the cell lines were grown to confluency in 175 $cm^2$ flasks. After trypsin treatment, the cells from this flask were used to seed two 850 $cm^2$ roller bottles and grown to confluency over a period of 4 days. The medium was then changed to serum free growth medium supplemented with 2mM sodium butyrate. The X'-hIgE-Fc was allowed to accumulate for 4 days before harvesting the medium.

In each case the X'-hIgE-Fc protein was purified from cell culture supernatant by affinity chromatography on an anti-hIgE-Fc mAb 7.12 Sepharose 4B matrix. The mAb used is described by *Sherr, J. Immunol. 142:481 (1989)*. The X'-hIgE-Fc protein was eluted from the column with 0.1M glycine-HCl, pH 2.5, and immediately neutralised with 1M Tris. All preparations were further purified by FPLC on a Pharmacia Superose 12HR 10/30 column in 130 mM NaCl, 200mM Tris-HCl, pH 8.4.

The purity of the product was assessed by SDS-polyacrylamide gel electrophoresis in 10% polyacrylamide gels using the technique described by *Laemmli, Nature 227:680 (1970)*. Gels were stained with 0.1% Coomassie blue. Four autoradiography gels were treated with a fluorographic enhancer (Amplify, Amersham Int., UK), dried under vacuum and exposed to Fuji-RX film. For Western blotting, proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), blocked in 1% casein in PBS, washed in PBS containing 0.01% Tween-20 and incubated with a mAb 7.12 $Fab_2$-alkaline phosphatase

conjugate at 0.2μg/ml for 2 hours at 21°C. After washing with PBS, the blot was developed with Fast red/naphthol phosphate (Sigma, UK).

30μg of purified triple mutant X'-hIgE-Fc (Ala 225, Gln 265, Gln 371) was used to determine the N-terminal sequence on an Applied Biosystems 470A gas phase sequencer with an on-line Applied Biosystems 120A HPLC for the analysis of PTH-amino acids as described by *Waterfield et al.* in *Handbook, Practical Protein Chemistry - A, A. Darbre, ed J Wiley & Sons, Chichester, UK, p411 (1986).*

Concentrations of X'-hIgE-Fc proteins in culture supernatants were monitored using an anti-IgE ELISA developed from a solid phase radio-immune assay described by *Vercelli et al., J Exp Med 169:1295 (1989).* This assay was essentially the same as that described by *Whittle et al., Protein Eng., 1:499 (1987),* except that polystyrene maxisorp 96 well microtitre plates (Dynatech Labs USA) were coated with 2μg/ml (100μl/well) of a mixture of anti-hIgE-Fc mAbs 7.12 and 4.15 (see *Sherr et al., J. Immunol., 142:481 (1989).* The monoclonal antibodies were coupled to the plate after 1 hour at room temperature in 0.1M sodium carbonate (pH 9.6) buffer. The plate was then washed three times with phosphate buffered saline (PBS) and blocked with 0.5% w/v casein in 0.1M sodium carbonate (pH 9.6) buffer. After six washes (PBS containing 0.025% v/v Tween 20), 100μl of the supernatants, diluted in PBS, were added to each well and incubated for 1 hour at room temperature. (The word "Tween" is a trade mark). The plate was washed as described above and 100μl of 1:1000 diluted rabbit anti-hIgE heavy chain-peroxidase conjugate (Dakopatts Ltd, Denmark) were added to each well to detect X'-hIgE-Fc proteins bound to the 7.12/4.15 mAbs and incubated for a further hour at room temperature. The wells were washed again and 100μl of substrate containing 0.1 mg/ml tetramethylbenzidine (TMB),

0.1M sodium citrate, pH 6.0, and 0.005% hydrogen peroxide were added to each well. After 30 minutes the optical density of each well was determined at 630nm in a Dynatech MR600 microtitre plate reader. Human myeloma IgE(PS) or hIgE-Fc was used as a standard.

The electrophoretic profiles in reducing gels of Figure 7 (lanes A1 to A4) show three species with apparent molecular weights of 42, 45 and 50kDa. The molecular weights are in accordance with the presence of a 38 kDa polypeptide (calculated from the amino acid composition) in each of the products plus different amounts of carbohydrate. The discrete sizes clearly reflect the addition of one, two or three pairs of oligosaccharide chains. Assuming that Asn 383 is not glycosylated and that the glycosylation occurs independently at the three sites, it is possible to make an identification of the products.

X'-hIgE-Fc(Ala 225, Gln 265, Gln 371) (Figure 7, lane A1) migrated as a single band of apparent molecular weight 42 kDa, which must represent X'-hIgE-Fc(Ala 225) with only the conserved carbohydrate at Asn 394. This is consistent with the observation that the X'-hIgE-Fc protein produced in E. coli migrates as a single band of slightly higher mobility (Figure 8, lanes 3 and 8). X'-hIgE-Fc(Ala 225, Gln 371) (Figure 7, lane A2) also formed a single band of apparent molecular weight of 45 kDa, which is inferred to represent polypeptides with two carbohydrate chains at Asn 394 and Asn 265. X'-hIgE-Fc(Ala 225, Gln 265) (Figure 7, lane A3), however, gave rise to a major component of 42 kDa, (which, like X'-hIgE-Fc(Ala 225, Gln 265, Gln 371), may have only the conserved carbohydrate at Asn 394) and a minor one of 45 kDa, glycosylated at both Asn 371 and Asn 394. Consistent with this interpretation, the wild type X'-hIgE-Fc (Ala 225) (Figure 7, lane A4) migrated as a doublet, with a major band of 45 kDa, which may represent

molecules glycosylated at the two preferred sites, Asn 265 and Asn 394, and a minor band of 50 kDa, which may represent molecules in which all three sites are glycosylated. A trace component of 42 kDa may represent X'-hIgE-Fc (Ala 225) glycosylated only at Asn 394. The results suggest that, of the two IgE-specific glycosylation sites, Asn 265 is fully glycosylated, but that Asn 371 is incompletely glycosylated in CHO cells.

The major species in X'-hIgE-Fc(Ala 225, Gln 371) and X'-hIgE-Fc(Ala 225, Gln 265, Gln 371) are both accompanied by a minor satellite band of slightly higher molecular weight. In previous studies of mouse IgG2a, it was found that microheterogeneity was eliminated by the treatment of cells with tunicamycin, which inhibits N-glycosylation according to *Leatherbarrow et al., Mol. Immunol., 22:407 (1985)*. This microheterogeneity was therefore attributed to glycosylation at the only site of glycosylation, homologous to Asn 394 in the $\epsilon$-chain of IgE. On this basis, it is believed that the satellite bands in X'-hIgE-Fc(Ala 225, Gln 371) and X'-hIgE-Fc(Ala 225, Gln 265, Gln 371) have a similar origin.

The separations in non-reducing gels (Figure 7, lanes B1 to B4) showed that all of the monomeric X'-hIgE-Fc(Ala 225) species are assembled into disulphide linked dimers (lanes 5-8). The size distributions suggest that when two forms of monomer are expressed they assemble into both homodimers and heterodimers. Thus, the assembly of the X'-hIgE-Fc(Ala 225, Gln 265) gives rise to two species (Figure 7, lane B3) of which the smaller is equivalent to the product of X'-hIgE-Fc(Ala 225, Gln 265, Gln 371) (Figure 7, lane B2) and thus by inference a homodimer of the same composition, lacking carbohydrate at both Asn 265 and Asn 371 in both $\epsilon$-chains. The larger component (Figure 7, lane B3) is smaller than the dimer formed by X'-hIgE-Fc(Ala 225,

Gln 371) (Figure 7, lane B1), which bears carbohydrate at both Asn 265 and Asn 394 of both chains. This larger component (Figure 7, lane B3) therefore corresponds to a heterodimer in which one chain is glycosylated only at position 394, and the other is glycosylated at both 394 and 371. The occurrence of only a single species of X'-hIgE-Fc(Ala 225, Gln 371) (Figure 7, lane B1) confirms that the Asn 265 is likely to be fully glycosylated.

Example 2

Production of X'-hIgE-Fc fragments by a stable cell line

Permanent CHO cell lines secreting either the wild type or double mutant X'-hIgE-Fc (Ala 225) fragments (X'-hIgE-Fc(Ala 225) and X'-hIgE-Fc(Ala 225, Gln 265, Gln 371)) were established, and cell lines secreting the X'-hIgE-Fc proteins with highest abundance were cloned by limiting dilution. In order to isolate the two products, cells were grown in roller bottles for 4 days at 37°C. In a preliminary experiment, the accumulation of each of the fragments was monitored by electrophoresis of harvest medium in SDS (sodium dodecyl sulphate) polyacrylamide gels and Western blotting. It was found that the X'-hIgE-Fc proteins accumulated to 2mg/l without the appearance of degradation products, as detected by the aforementioned ELISA assay (standardised against purified myeloma IgE(PS)), in 4 days. After this time, the concentration of the intact protein decreased, and specific degradation products began to accumulate.

Example 3

An NS-0 cell line secreting X'-hIgE-Fc(Ala 225, Gln 265, Gln 371) was also established. For expression in NS-0 cells, the X'-hIgE-Fc(Ala 225, Gln 265, Gln 371) mutant construct was subcloned into a pEE6 based expression vector containing glutamine synthetase cDNA as a selectable marker according to the technique described by *Bebbington et al.*,

*Biotechnology 10:169 (1992)*. $10^7$ NS-O cells in the exponential phase of growth were pelleted at 1500 RPM for 5 minutes and were washed twice with a solution of ice-cold phosphate-buffered saline (0.15 M NaCl, 0.05 M phosphate buffer, pH 7.6). The cells were resuspended in a final volume of 0.7 ml of ice-cold PBS. From this stage onwards of the transfection procedure the cells were maintained on ice. 40 µg of the linearised expression vector was mixed together with the suspension of NS-O cells in a 0.4 cm electroporation cuvette (Bio-Rad) and left on ice for 5 minutes. Electroporation of the cells was performed using a Gene Pulser (Bio-Rad) using two consecutive 0.1 sec. pulses of 1500 V at a capacitance of 3 µF. The cuvette was returned to ice for a further 2-5 minutes before the electroporated cells were added to 40 ml of non-selective media. 30 ml of this was plated out over three 96 well culture dishes, the rest was diluted a further three times by a factor of four. Each dilution was plated out over three 96 well culture dishes. The cells were allowed to recover overnight and the next day 100 µl of gDMEM selective medium was added to the cells. Resistant colonies appeared after about four weeks after the addition of selection to the transfected cells. Single colonies able to grow in glutamine-free media were screened using the anti-human IgE ELISA (see below) and the best producers were expanded.

A suspension adapted form of this line, growing in a serum replacement medium, was able to accumulate up to approximately 100mg of product (i.e. X'-hIgE-Fc(Gln 265, Gln 371)) per litre of culture supernatant (as determined by the aforementioned anti-human IgE ELISA, standardised against purified X'-hIgE-Fc(Gln 265, Gln 371)).

The X'hIgE-Fc proteins were purified from the harvest medium by affinity chromatography on an anti-hIgE-Fc matrix and examined for purity by electrophoresis on SDS

polyacrylamide gels (Figure 8). Under non-reducing conditions, essentially all of the protein was recovered in the form of disulphide-linked dimers. Under reducing conditions, monomers of the expected size were found.

Example 4

To scale up the production of protein from NS-0 cell lines required the adaptation of the cell line to suspension culture. The NS-0 cell line was also adapted to growth in gDMEM containing a serum replacement (Celltech Ltd). To adapt transfected cells to suspension culture the cells were grown in 490 $cm^2$ roller bottles and were regularly passaged before they reached confluency. Adaptation to serum replacement media was achieved by gradually reducing the proportion of FCS in the media each time the cells, growing in suspension, were passaged. The supernatant media was harvested from the cells at the time that they became confluent.

Example 5

Nine amino acids were sequenced from the N-terminus of X'-hIgE-Fc (Gln 265, Gln 371) and the sequence was determined to be:

[SEQ ID No: 3]

Asp Ile Val Ala Ser Xaa Asp Phe Thr

The sixth residue, Xaa, which should have been Arg, could not be unambiguously assigned. A comparison of the observed and predicted sequences (Figure 3) indicates that the fragment has the correct N-terminal amino acid, consistent with accurate leader sequence processing. Thus Cys 225 has been successfully replaced by Ala. The rest of the determined N-terminal sequence is as expected.

Example 6

IgE(WT), i.e. IgE isolated from a myeloma of a particular patient (designated "WT"), or the wild type X'-hIgE-Fc or mutant X'-hIgE-Fc protein was labelled with

[$^{125}$I] iodine to a specific activity of 0.5 - 1 x $10^9$ cpm/mg protein using chloramine T (see *McConahey et al., Methods in Enzym., 70:213 (1980)*). The fraction of functional X'-hIgE-Fc protein and IgE(WT) was determined from the percentage of molecules in the purified samples of X'-hIgE-Fc and X'-hIgE-Fc(Gln 265, Gln 371) bound to an excess of cells of a stable CHO cell line expressing recombinant human FcεRI (CHO-hFcεRI) (see *Wang et al., J. Exp. Med., 175:1353 (1992)*. The method of *Kulczycki Jr. et al., J. Exp. Med., 140:1676 (1974)* was used. The concentrations of the X'-hIgE-Fc protein and IgE(WT) used in all binding assays was corrected for the fraction of functional molecules, typically in the range of 30 - 70%.

The affinity of the ligands, i.e. X'-hIgE-Fc, its mutants and IgE(SF25), for FcεRI was measured from the forward and reverse constants for the rates of reaction with cell-bound FcεRI. The value of $k_{+1}$ was determined by measuring the concentration of ligand bound to cells as a function of time (see *Kulczycki, Jr. et al., J. Exp. Med., 140:1676 (1974)*). A curve fitting program (ORIGIN Ver. 3, Microcal Software, MA,USA) was used to obtain the best fit to the equation below, relating the bound ligand to $k_{+1}$ and $k_{-1}$:

$$[RL] = \left[\frac{k_{+1}[L][R_T]}{k_{+1}[L] + k_{-1}}\right] - \left[\frac{k_{+1}[L][R_T]}{k_{+1}[L] + k_{-1}}\right] e^{-(k_{+1}[L]+k_{-1})t}$$

where [RL] is the concentration of receptor-ligand complex, $[R_T]$ is the total receptor concentration, and [L] is the total ligand concentration. The value of $k_{-1}$ was determined by the method of *Kulczycki et al., J. Exp. Med. 140:1676 (1974)*.

The kinetics of binding of X'-hIgE-Fc and X'-hIgE-Fc(Gln 265, Gln 371) to FcεRI were measured using the CHO-hFcεRI cell line. For comparison the kinetics of

the CHO-hFcεRI cell line. For comparison the kinetics of binding of IgE(SF25) were also measured. The rates of association for IgE(WT), X'-hIgE-Fc, and X'-hIgE-Fc(Gln 265, Gln 371) were 3.1 ± 0.3 x $10^5$ $M^{-1}s^{-1}$ (n = 3), 8.1 ± 0.8 x $10^5$ $M^{-1}s^{-1}$ (n = 6), and 9.9 ± 1.1 x $10^5$ $M^{-1}s^{-1}$ (n = 4). The corresponding rates of dissociation were 0.9 ± 0.2 x $10^{-5}$ $M^{-1}s^{-1}$ (n = 6), 1.0 ± 0.1 x $10^{-5}s^{-1}$ (n = 6) and 2.0 ± 0.2 x $10^{-5}$ $M^{-1}s^{-1}$ (n = 6) (Table I). The $K_a$ values calculated from the average $k_{+1}$ and $k_{-1}$ values, are listed in Table I.

Experiments were also carried out to measure binding of X'-hIgE-Fc(Gln 265, Gln 371) to FcεRII. 5 x $10^5$ RPMI 8866 cells were added to mixtures of [$^{125}$I]-labelled IgE, wild type X'-hIgE-Fc, or mutant X'-hIgE-Fc(Gln 265, Gln 371) at 2μg/ml (10.2 nM and 27.8 nM respectively) and increasing amounts of the unlabelled sample. The amount of cell-bound labelled ligand was determined as described above for the FcεRI binding study. The $K_a$ values were calculated from the $IC_{50}$ values, determined by non-linear regression of the data (using the GraphPad-Inplot software package), using the following equation:

$$K_i \text{ (inhibition constant)} = IC_{50}/(1 + [L^*]/K_d);$$

the $K_i$ can be assumed to be the same as the $K_d$ if the labelled and unlabelled ligand are the same. Therefore

$$K_a = (IC_{50} - [L^*])^{-1}.$$

[L*] = concentration of labelled ligand, corrected for the percentage bindability (as described above).

TABLE 1

| Protein assayed | FcεRI | | | FcεRII |
|---|---|---|---|---|
| | $k_{+1}$ ($M^{-1}s^{-1}$) | $k_{-1}$ ($s^{-1}$) | $K_a$ ($M^{-1}$) | $K_a$ ($M^{-1}$) |
| X'-hIgE-Fc | $3.1\pm0.3 \times 10^5$ (3) | $0.9\pm0.2 \times 10^{-5}$ (6) | $3.4 \times 10^{10}$ | $7.3\pm0.6 \times 10^7$ |
| hIgE | $6.1\pm0.8 \times 10^5$ (6) | $1.0\pm0.1 \times 10^{-5}$ (6) | $6.1 \times 10^{10}$ | $4.1\pm1.5 \times 10^6$ |
| X'-hIgE-Fc (Gln 265, Gln 371) | $9.9\pm1.1 \times 10^5$ (4) | $2.0\pm0.2 \times 10^{-5}$ (6) | $5.0 \times 10^{10}$ | $3.2\pm2.4 \times 10^7$ |

Notes:

1. $k_a$ was calculated as $k_{+1}/k_{-1}$.
2. The number of experiments performed is given in brackets.

Example 7

$K_a$ values for the association of IgE, wild type X'-hIgE-Fc and the X'-hIgE-Fc(Gln 265, Gln 371) mutant with the RPMI 8866 cell's FcεRII receptor were obtained from competition curves. The $K_a$ values calculated from the competition curves are $4.1 \times 10^6$ $M^{-1}$ and $3.2 \times 10^7$ $M^{-1}$ for X'-hIgE-Fc and X'-hIgE-Fc(Gln 265, Gln 371) respectively, as compared with the value of $7.3 \times 10^7$ $M^{-1}$ for IgE(WT), averaged over four independent experiments, each performed in duplicate.

Example 8

Effector function of the wild type X'-hIgE-Fc and double mutant recombinant X'-hIgE-Fc(Gln 265, Gln 371) was tested by the ability of anti-IgE to trigger histamine release from human basophils sensitised with the X'-hIgE-Fc proteins. Preliminary experiments showed that maximal histamine release was obtained when cells were sensitised with IgE(PS) or X'-hIgE-Fc proteins at concentrations of approximately 30nM or higher. The histamine release induced by various dilutions of anti-IgE from basophils sensitised with 60 nM IgE and the X'-hIgE-Fc proteins was studied.

Basophils sensitised with IgE and the X'-hIgE-Fc proteins showed similar response curves following anti-IgE stimulation, whereas only marginal release was observed from non-sensitised basophils. The results are plotted in Figure 9. Histamine release induced by an optimal dilution (1:1000) of anti-IgE from cells fully sensitised with IgE(PS), X'-hIgE-Fc and X'-hIgE-Fc(Gln 265, Gln 371) was 57.5 ± 4.5%, 67.7 ± 5.1% and 62.0 ± 10.5% (mean ± SD, n=4, 3, 3), respectively.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Dr. C. T. Eyles, c/o W. P. Thompson & Co.

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(I) TELEX: 268801

(ii) TITLE OF INVENTION: Polypeptide competitors for IgE receptor sites on cells.

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: GB 9324013.3

(B) FILING DATE: 22-NOV-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 323 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ser Arg Asp Phe Thr Pro Pro Thr Val Lys Ile Leu Gln Ser Ser Cys
1 5 10 15

Asp Gly Gly Gly His Phe Pro Pro Thr Ile Gln Leu Leu Cys Leu Val
20 25 30

Ser Gly Tyr Thr Pro Gly Thr Ile Asn Ile Thr Trp Leu Glu Asp Gly
35 40 45

Gln Val Met Asp Val Asp Leu Ser Thr Ala Ser Thr Thr Gln Glu Gly
50 55 60

Glu Leu Ala Ser Thr Gln Ser Glu Leu Thr Leu Ser Gln Lys His Trp
65 70 75 80

Leu Ser Asp Arg Thr Tyr Thr Cys Gln Val Thr Tyr Gln Gly His Thr
85 90 95

Phe Glu Asp Ser Thr Lys Lys Cys Ala Asp Ser Asn Pro Arg Gly Val
100 105 110

Ser Ala Tyr Leu Ser Arg Pro Ser Pro Phe Asp Leu Phe Ile Arg Lys
115 120 125

Ser Pro Thr Ile Thr Cys Leu Val Val Asp Leu Ala Pro Ser Lys Gly
130 135 140

Thr Val Asn Leu Thr Trp Ser Arg Ala Ser Gly Lys Pro Val Asn His
145 150 155 160

Ser Thr Arg Lys Glu Glu Lys Gln Arg Asn Gly Thr Leu Thr Val Thr
165 170 175

Ser Thr Leu Pro Val Gly Thr Arg Asp Trp Ile Glu Gly Glu Thr Tyr
180 185 190

Gln Cys Arg Val Thr His Pro His Leu Pro Arg Ala Leu Met Arg Ser
195 200 205

Thr Thr Lys Thr Ser Gly Pro Arg Ala Ala Pro Glu Val Tyr Ala Phe
210 215 220

Ala Thr Pro Glu Trp Pro Gly Ser Arg Asp Lys Arg Thr Leu Ala Cys
225 230 235 240

Leu Ile Gln Asn Phe Met Pro Glu Asp Ile Ser Val Gln Trp Leu His
245 250 255

Asn Glu Val Gln Leu Pro Asp Ala Arg His Ser Thr Thr Gln Pro Arg
260 265 270

Lys Thr Lys Gly Ser Gly Phe Phe Val Phe Ser Arg Leu Glu Val Thr
275 280 285

Arg Ala Glu Trp Glu Gln Lys Asp Glu Phe Ile Cys Arg Ala Val His
290 295 300

Glu Ala Ala Ser Pro Ser Gln Thr Val Gln Arg Ala Val Ser Val Asn
305 310 315 320

Pro Gly Lys

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1308 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GTCTGCTCCA GGGACTTCAC CCCGCCCACC GTGAAGATCT TACAGTCGTC CTGCGACGGC 60

GGCGGGCACT TCCCCCCGAC CATCCAGCTC CTGTGCCTCG TCTCTGGGTA CACCCCAGGG 120

ACTATCAACA TCACCTGGCT GGAGGACGGG CAGGTCATGG ACGTGGACTT GTCCACCGCC 180

TCTACCACGC AGGAGGGTGA GCTGGCCTCC ACACAAAGCG AGCTCACCCT CAGCCAGAAG 240

CACTGGCTGT CAGACCGCAC CTACACCTGC CAGGTCACCT ATCAAGGTCA CACCTTTGAG 300

GACAGCACCA AGAAGTGTGC AGGTACGTTC CCACCTGCCC TGGTGGCCGC CACGGAGGCC 360

AGAGAAGAGG GGCGGGTGGG CCTCACACAG CCCTCCGGTG TACCACAGAT TCCAACCCGA 420

GAGGGGTGAG CGCCTACCTA AGCCGGCCCA GCCCGTTCGA CCTGTTCATC CGCAAGTCGC 480

CCACGATCAC CTGTCTGGTG GTGGACCTGG CACCCAGCAA GGGGACCGTG AACCTGACCT 540

GGTCCCGGGC CAGTGGGAAG CCTGTGAACC ACTCCACCAG AAAGGAGGAG AAGCAGCGCA 600

ATGGCACGTT AACCGTCACG TCCACCCTGC CGGTGGGCAC CCGAGACTGG ATCGAGGGGG 660

AGACCTACCA GTGCAGGGTG ACCCACCCCC ACCTGCCCAG GGCCCTCATG CGGTCCACGA 720

CCAAGACCAG CGGTGAGCCA TGGGCAGGCC GGGGTCGTGG GGGAAGGGAG GGAGCGAGTG 780

AGCGGGGCCC GGGCTGACCC CACGTCTGGC CACAGGCCCG CGTGCTGCCC CGGAAGTCTA 840

TGCGTTTGCG ACGCCGGAGT GGCCGGGGAG CCGGGACAAG CGCACCCTCG CCTGCCTGAT 900

CCAGAACTTC ATGCCTGAGG ACATCTCGGT GCAGTGGCTG CACAACGAGG TGCAGCTCCC 960

GGACGCCCGG CACAGCACGA CGCAGCCCCG CAAGACCAAG GGCTCCGGCT TCTTCGTCTT 1020

CAGCCGCCTG GAGGTGACCA GGGCCGAATG GGAGCAGAAA GATGAGTTCA TCTGCCGTGC 1080

AGTCCATGAG GCAGCGAGCC CCTCACAGAC CGTCCAGCGA GCGGTGTCTG TAAATCCCGG 1140

TAAATGACGT ACTCCTGCCT CCCTCCCTCC CAGGGCTCCA TCCAGCTGTG CAGTGGGGAG 1200

GACTGGCCAG ACCTTCTGTC CACTGTTGCA ATGACCCCAG GAAGCTACCC CCAATAAACT 1260

GTGCCTGCTC AGAGCCCCAG TACACCCATT CTTGGGAGCG GGCAGGGC 1308

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Asp Ile Val Ala Ser Xaa Asp Phe Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Lys Thr Phe Ser Val Cys Ser Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AAAACCTTCA GCGTCTGCTC CAGGGAC 27

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Cys Asp Ile Val Ala Ser Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TGTGATATCG TCGCCTCCAG GGAC 24

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGGACTATCC AGATCACCTG G 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCAGGTGATC TGGATAGTCC C 21

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGGACCGTGC AGCTGACCTG G 21

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCAGGTCAGC TGCACGGTCC C 21

CLAIMS

1. A mutated glycosylated polypeptide which includes at least a part of the hIgE-Fc chain of sufficient length to bind to FcεRI and/or FcεRII receptor sites on human cells wherein Cys 225 has been mutated by replacement with another amino acid residue or has been deleted, optionally together with Val 224 and Ser 226 or with Val 224 Ser 226 and Arg 227 or with Val 224 Ser 226 Arg 227 and Asp 228, and wherein at least one of the sites Asn 394 and, if present, Asn 265 and/or Asn 371 bears a glycoside chain.

2. A polypeptide which binds to human immunoglobulin E (hIgE) receptor sites on cells and which is of the formula:

$AA_n$-hIgE-Fc (W225, X265, Y371, Z394)

wherein:

AA represents an amino acid residue which may be the same as or different from any other group AA which may be present in the molecule;

n represents zero or an integer from 1 to about 10; and

hIgE-Fc (W225, X265, Y371, Z394) represents a mutant version of the hIgE-Fc chain with a mutation or deletion at least at position 225; wherein

W225 represents deletion of Val 224 and Cys 225, of Val 224, Cys 225 and Ser 226, or of Val 224, Cys 225, Ser 226 and Arg 227, or of Val 224, Cys 225, Ser 226, Arg 227 and Asp 228, or represents the residue of an amino acid at position 225 other than cysteine;

X265 is the residue of an amino acid at position 265;

Y371 is the residue of an amino acid at position 371; and

Z394 is the residue of an amino acid at position

394; and wherein at least one of X265, Y371 and Z394 may be an asparagine residue which may be glycosylated; or a fragment of such a polypeptide which lacks up to 10 terminal amino acid residues of the Cε4 domain at the carboxy end of the chain.

3. A polypeptide according to claim 2, wherein W225 represents an alanine residue.

4. A polypeptide according to claim 2 or claim 3, wherein X265 represents a glutamine residue.

5. A polypeptide according to any one of claims 2 to 4, wherein Y371 represents a glutamine residue.

6. A polypeptide according to any one of claims 2 to 5, wherein Z394 represents a glutamine residue.

7. A polypeptide according to claim 2, which is selected from:

$AA_n$-hIgE-Fc (Ala 225)
$AA_n$-hIgE-Fc (Ala 225, Gln 265)
$AA_n$-hIgE-Fc (Ala 225, Gln 371)
$AA_n$-hIgE-Fc (Ala 225, Gln 394)
$AA_n$-hIgE-Fc (Ala 225, Gln 265, Gln 371)
$AA_n$-hIgE-Fc (Ala 225, Gln 265, Gln 394)
$AA_n$-hIgE-Fc (Ala 225, Gln 371, Gln 394)
$AA_n$-hIgE-Fc (Ala 225, Gln 265, Gln 371, Gln 394)

8. A polypeptide according to any one of claims 2 to 7, wherein $AA_n$ represents an inert polypeptide sequence.

9 A polypeptide according to any one of claims 2 to 8, wherein $AA_n$ represents Asp-Ile-.

10. A polypeptide according to any one of claims 2 to 9, wherein the N-terminal sequence of the group hIgE-Fc (W225, X265, Y371, Z394) has the structure:

Asp Ile Val Ala Ser Xaa Asp Phe Thr

where Xaa is the residue of an amino acid.

11. A polypeptide according to claim 10, wherein Xaa is an arginine residue.

12. A polypeptide according to claim 2, wherein W225 represents the deletion of Val 224 and Cys 225, of Val 224, Cys 225 and Ser 226, or of Val 224, Cys 225, Ser 226 and Arg 227.

13. DNA coding for or a polypeptide according to any one of claims 1 to 12.

14. A host vector containing cDNA coding for a polypeptide according to any one of claims 1 to 12.

15. A mammalian cell line containing DNA according to claim 13.

16. A pharmaceutical preparation comprising a polypeptide according to any one of claims 1 to 12 and a carrier therefor.

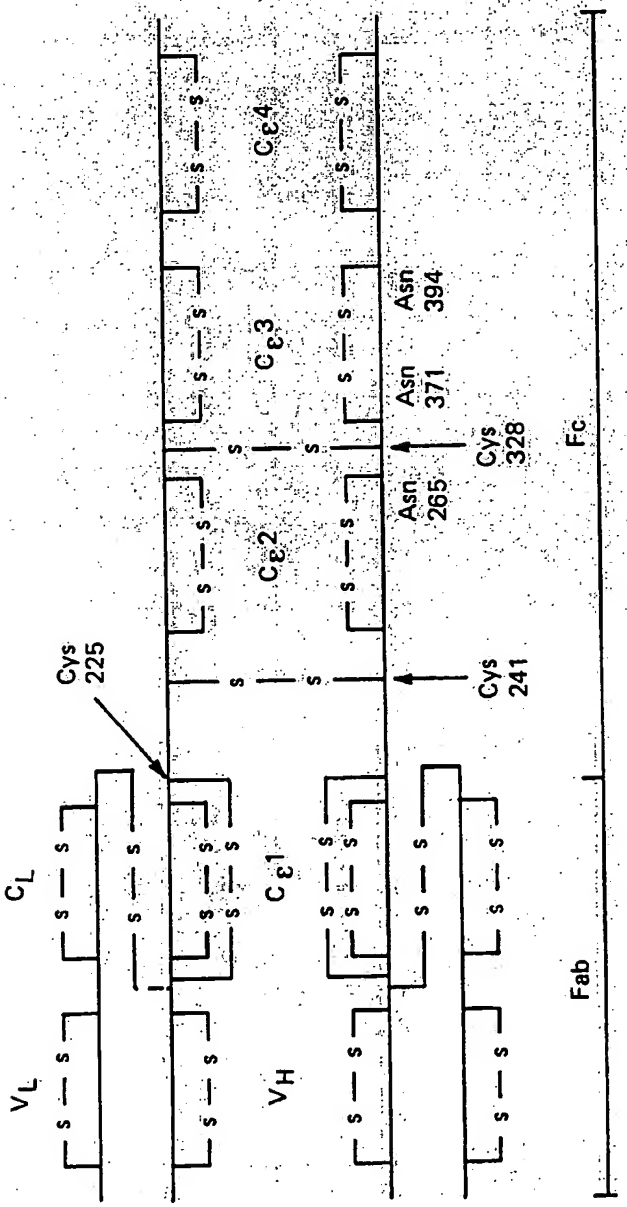
Cε4
Cε3
Cε2
Cε1
CL
VL
VH
Cys 225
Cys 241
Asn 265
Cys 328
Asn 371
Asn 394
Fc
Fab

FIG. 1

**Natural $C_\varepsilon 1/C_\varepsilon 2$ junction:**

5'

. . . LYS THR PHE SER / VAL CYS SER ARG ASP. . .
. . . AAA ACC TTC AGC / GTC TGC TCC AGG GAC. . .

FIG. 2

**Leader sequence-adapter/$C_\varepsilon 2$ junction:**

5'

MET. . . .CYS ASP ILE / VAL ALA SER ARG ASP. . .
ATG. . . .TGT GAT ATC / GTC GCC TCC AGG GAC. . .

225

Eco RV

FIG. 3

Asn 265 ⟶ Gln 265:

5'

GGG ACT ATC CAG ATC ACC TGG
CCC TGA TAG GTC TAG TGG ACC

FIG. 4

Asn 371 ⟶ Gln 371:

5'

GGG ACC GTG CAG CTG ACC TGG
CCC TGG CAC GTC GAC TGG ACC

FIG. 5 RECTIFIED SHEET (RULE 91)
ISA/EP

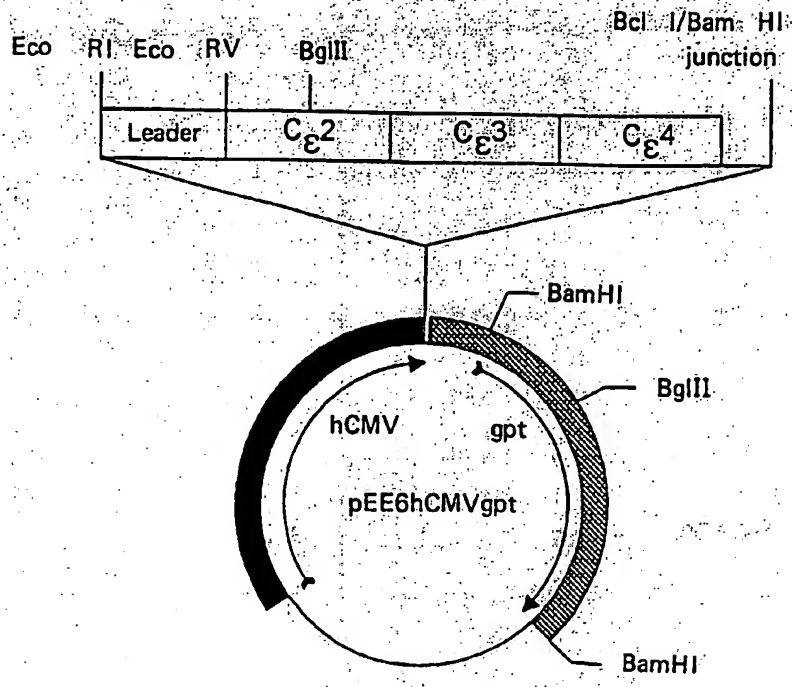
Eco RI
Eco RV
BglII
Bcl I/Bam HI junction
Leader
$C_\varepsilon 2$
$C_\varepsilon 3$
$C_\varepsilon 4$
BamHI
BglII
hCMV
gpt
pEE6hCMVgpt
BamHI

FIG. 6

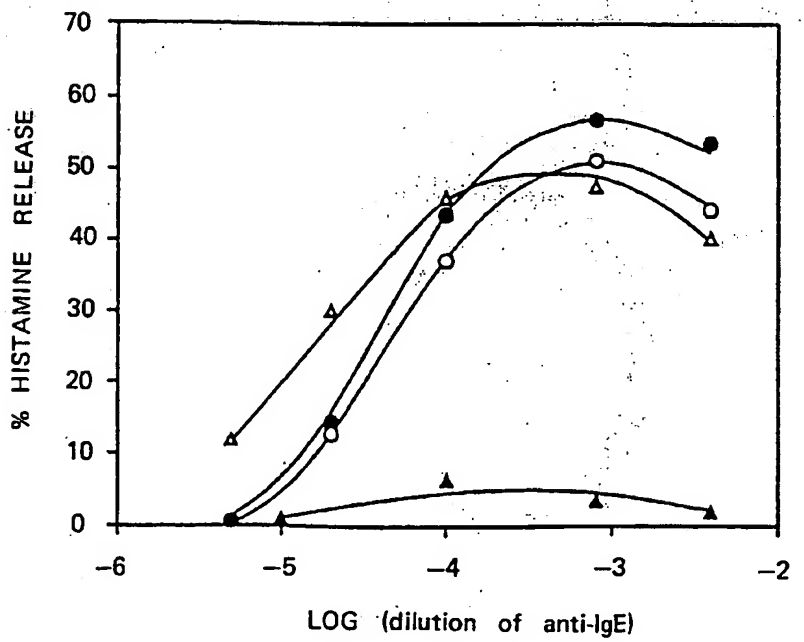
% HISTAMINE RELEASE
70
60
50
40
30
20
10
0
−6
−5
−4
−3
−2
LOG (dilution of anti-IgE)

FIG. 9

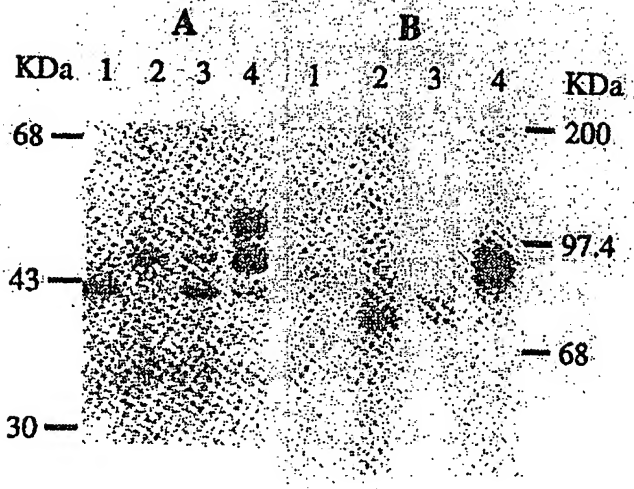
A
B
KDa 1 2 3 4 1 2 3 4 KDa
68
43
30
200
97.4
68

FIG. 7

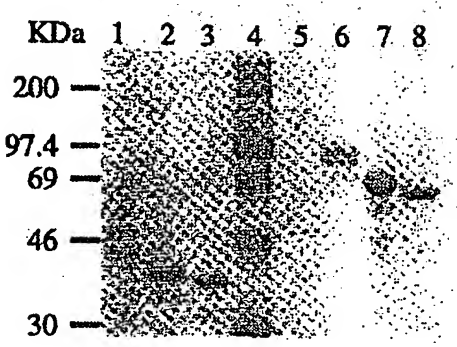
KDa 1 2 3 4 5 6 7 8
200
97.4
69
46
30

FIG. 8

# INTERNATIONAL SEARCH REPORT

International Appli n No
PCT/GB 94/02561

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/13 C07K16/28 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO,A,93 04173 (GENETECH, INC.; US) 4 March 1993<br>see the whole document<br>--- | 1-16 |
| A | JOURNAL OF BIOLOGICAL CHEMISTRY.,<br>vol.268, no.18, 25 June 1993, BALTIMORE US<br>pages 13118 - 13127<br>BASU, M. ET AL.; 'Purification and characterization of human recombinanat IgE-Fc fragments that bind to the human high affinity IgE receptor'<br>cited in the application<br>see the whole document<br>---<br>-/-- | 1-16 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 11 April 1995 | 02-05-1995 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+ 31-70) 340-3016 | Nauche, S |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International Appli n No
PCT/GB 94/02561

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | JOURNAL OF IMMUNOLOGY., vol.141, no.9, 1 November 1988, BALTIMORE US pages 3128 - 3134 CHRETIEN I;HELM BA;MARSH PJ;PADLAN EA;WIJDENES J;BANCHEREAU J; 'A monoclonal anti-IgE antibody against an epitope (amino acids 367-376) in the CH3 domain inhibits IgE binding to the low affinity IgE receptor (CD23).' see the whole document --- | 1-16 |
| A | BIOCHEMICAL SOCIETY TRANSACTIONS., vol.21, November 1993 pages 968 - 972 BEAVIL, A.J. ET AL.; 'Structural basis of the IgE-Fcepsilon RI interaction' see the whole document ----- | 1-16 |

2

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Appli n No
PCT/GB 94/02561

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO-A-9304173 | 04-03-93 | AU-A- 2498192 | 16-03-93 |
| | | EP-A- 0602126 | 22-06-94 |
| | | JP-T- 6509944 | 10-11-94 |